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(54) Title: QUANTIFICATION OF BACTERIA USING A NUCLEIC ACID HYBRIDIZATION ASSAY

(57) Abstract

This invention provides for a method of quantifying bacteria using a bacterial specific nucleic acid probe which is complementary to a unique and highly conserved region of the 16S ribosomal RNA (rRNA) of bacteria. This probe permits the rapid detection of 16S rRNA in a sample and by comparison with known standards, one can estimate the total bacterial count in the sample. The method is accurate and reproducible and conducted at temperatures of between about 12 ° to about 14 °C.

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QUANTIFICATION OF BACTERIA USING A NUCLEIC ACID HYBRIDIZATION ASSAY

5

BACKGROUND OF THE INVENTION

Field of the Invention

10 This invention provides for a method of quantifying
bacteria using a bacterial specific nucleic acid probe which is
complementary to a unique, open and highly conserved region of
the 16S ribosomal RNA (rRNA) of bacteria. This probe permits
the rapid detection of 16S rRNA in a sample and by comparison
with known standards, one can estimate the total bacterial
15 count in the sample. The method is accurate, reproducible and
conducted at room temperature.

Information Disclosure

20 The use of signal intensity of nucleic acid hybridization assay to estimate total nucleic acid present in a sample is known. Gowans, E.J., Jilbert, A.R. and Burrell, C.J., Detection of Specific DNA and RNA Sequences in Tissues and Cells By *In Situ* Hybridization (Chapter 5, 1989) in Nucleic Acid Probes, Ed. Symons, R.H., CRC Press, Inc. Boca Raton, 25 Florida; and Anderson, M.L.M. and Young, B.D., 1987, Quantitative Filter Hybridisation (Chapter 4) in Nucleic Acid Hybridisation a Practical Approach, Eds. B.D. Hames and S.J. Higgins, IRL Press, Washington D.C. USA.

Universal probes for the detection of bacteria are known. Giovannoni, S.J. et al., 1988, Phylogenetic Group-specific Oligodeoxynucleotide Probes for Identification of Single Microbial Cells, *J. Bact.* 170(2):720-726 and Chuba, P.J. et al., 1988, Synthetic Oligodeoxynucleotide Probes for the Rapid Detection of Bacteria Associated with Human Periodontitis, *J. Gen. Microbiol.* 134:1931-1938. Oligonucleotides reflecting the UP9A region were described by Woese, C.R., et al., (1975), Conservation of primary structure

in 16S ribosomal RNA, *Nature* 254:83-85 (see Table 1, oligos 47, 49 and 51) and WO 88/03957 (see page 105).

The use of total bacterial count to diagnose periodontal disease is not a presently accepted practice.

5 Socransky, S.S. et al., *The Microbiota of the Gingival Crevice Area of Man-I Total Microscopic and Viable Counts and Counts of Specific Organisms*, *Arch. Oral. Biol* 8:275-280 and Moore, W.E.C., 1987, *Microbiology of Periodontal Disease*, *J. Periodontal Res.* 22:335-341.

10 Microbial counts were used to determine the effectiveness of tetracycline for prevention of periodontal disease by the Forsyth Center and reported in *J. of Dental Res.* Annual Session, March 15-19, 1989, Vol. 68, page 197, Abstract Nos. 122-124.

15

SUMMARY OF THE INVENTION

This invention provides for a method of measuring the quantity of bacteria in a biological sample which comprises lysing the bacteria in the sample and contacting the lysate 20 under hybridization conditions with an oligonucleotide probe having a sequence of 5'CTGCTGCCTCCCGTAGGAGT3'. The phrase "an oligonucleotide probe having a sequence of 5'CTGCTGCCTCCCGTAGGAGT3' is meant to include functional equivalents of this sequence. Such equivalents are described 25 in greater detail below but embrace nucleic acid analogs and minor mismatched oligonucleotides, such that the probes will bind specifically to the target region on the 16S rRNA to which the claimed sequence is complementary.

The term "lysate" refers to solutions containing 30 bacterial nucleic acid. A lysate would include crude mixtures of disrupted bacteria, semi-purified solutions and purified solutions of bacterial nucleic acid.

The claimed probe may either be a capture probe or signal probe. Capture probes are unlabelled probes which bind 35 to target nucleotides and subsequently capture the target to a solid support. Signal probes are adapted to be used for the generation of a signal, for example a probe with a avidin moiety.

Samples can be obtained from any biological source including a human being and particularly from blood, mouth region or anogenital region.

5 The method can be further enhanced by the addition of at least one additional nucleic acid probe which is species specific, genus-specific or strain-specific. These additional probes can provide qualitative information in addition to quantification of bacteria.

10 This invention also provides for diagnostic kits utilizing the above technology.

DETAILED DESCRIPTION

This invention relates to the use of a unique sequence of nucleic acid, designated UP9A, which provides 15 universal binding to the 16S rRNA (see Table 1). This sequence is particularly unique to bacterial rRNA and does not significantly hybridize to human nucleic acid. In addition this sequence is located in a region of the ribosome where it is available for hybridization with only minimal disturbance of 20 the secondary structure of the rRNA. Thus the quantification assays can be done without heat denaturation of the sample. Target sequences having this characteristic are termed "open" regions because of their relative availability for hybridization.

25 Quantification of bacteria is dependent upon the ability of the assay to react in a predictable manner to increasing amounts of rRNA. The UP9A probe reacts in predictable manner, typically by offering a direct and linear response to increasing amounts of bacterial rRNA. By 30 preparation of and by comparison to appropriate standards, one can readily quantify the total bacterial count in a sample using the disclosed invention.

It is anticipated that the invention will find 35 application in an unlimited number of clinical and industrial settings where the rapid monitoring of bacterial counts are useful. Bacterial counts are of particular use in diagnosing disease states where high bacterial counts are indicative of the particular disease state. For example bacterial counts are

useful in diagnosing periodontal disease, stomach ulcers, bacteremia, and urinary tract infections. In addition, rapid bacterial quantification is often desirable during food preparation and fermentation processes.

5

Table 1. Examples of Universal Oligonucleotides for 16S bacterial ribosomes.

	<u>16S rRNA oligonucleotide probes</u>	<u>E. coli base position</u>
10		
	UP7B 5'GTATTACCGCGGCTGCTG3'	519-536
	UP3A 5'TGACGGCGGTGTGTACAA3'	1390-1408
	UP9A 5'CTGCTGCCTCCCGTAGGAGT3'	338-357
15		

Obtaining Oligonucleotide, UP9A

The degree of complementarity (homology) required for detectable binding of UP9A probes with the rRNA of bacteria will vary in accordance with the stringency of the hybridization medium and/or wash medium. The degree of complementarity will optimally be 100 percent; however, it should be understood that minor variations between the rRNA and UP9A may be compensated for by reducing the stringency of the hybridization and/or wash medium as described below. Thus, despite the lack of 100 percent complementarity under reduced conditions of stringency, functional probes having minor base differences from their rRNA targets are possible. Therefore, under hybridization conditions of reduced stringency, it may be possible to slightly modify the UP9A probe while maintaining an acceptable degree of specificity to quantify total bacteria present.

The UP9A oligonucleotide may be a compound of RNA or DNA. In addition, analogs of nucleosides may be substituted for naturally occurring nucleosides. The advantage of analogs would include greater stability, resistance to nuclease activity and ease of signal attachment. The term UP9A is intended to embrace all functionally equivalent species.

Equivalent UP9A probes may also consist of the given sequence, concatemers of the sequence, or probes flanked by about 10 or less bases of any degree of complementarity to the native sequences flanking the UP9A complementary region of bacterial rRNA.

5 UP9A probe may be chemically synthesized using commercially available methods and equipment. For example, the solid phase phosphoramidite method can be used to produce short probes of between 15 and 50 bases. For this invention, it is 10 preferred to chemically synthesize short DNA probes using the Model 380B DNA Synthesizer from Applied Biosystems, Foster City, California, using reagents supplied by the same company.

15 To obtain large quantities of UP9A probes, one can also clone the desired sequence using traditional cloning methods, such as described in Maniatis, T., et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1982, or one can produce the probes by chemical synthesis using commercially available DNA synthesizers. An example of cloning 20 would involve insertion of the cDNA for the ribosomal RNA into a replication vector, such as pBR322, M13, or into a vector containing the SP6 promotor (e.g., generation of single-stranded RNA using SP6 RNA polymerase), and transformation of a bacterial host. The DNA probes can be purified from the host 25 cell by lysis and nucleic acid extraction, treatment with selected restriction enzymes, and further isolation by gel electrophoresis.

30 The use of polymerase chain reaction technology can also be used to obtain large quantities of the UP9A probe. (See U.S. Patent No. 4,683,202.)

35 The UP9A probe can be used as a capture probe in a sandwich-type assay where the bacterial rRNA is the target nucleic acid and a second or other signal probes facilitates detection. Table 1 provides UP7B and UP3A which are useful as additional universal probes for signal detection.

UP9A probes can also serve as signal probes. Signal probes may be labeled by any one of several methods typically used to detect the presence of hybrid polynucleotides. The most common method of detection is the use of autoradiography

with ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P labeled probes or the like. The choice of radioactive isotope depends on research preferences due to ease of synthesis, stability and half lives of the selected isotopes. Other labels include ligands which bind to antibodies labeled with fluorophores, chemiluminescent agents, and enzymes. Alternatively, probes can be conjugated directly with labels such as fluorophores, chemiluminescent agents or enzymes. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

The choice of label dictates the manner in which the label is bound to the probe. Radioactive probes are typically made using commercially available nucleotides containing the desired radioactive isotope. The radioactive nucleotides can be incorporated into probes, for example, by using DNA synthesizers, by nick translation with DNA polymerase I, by tailing radioactive DNA bases to the 3' end of probes with terminal deoxynucleotidyl transferase, by treating single-stranded M13 plasmids having specific inserts with the Klenow fragment of DNA polymerase in the presence of radioactive deoxynucleotides (dNTP), by transcribing from RNA templates using reverse transcriptase in the presence of radioactive deoxynucleotides (dNTP), or by transcribing RNA from vectors containing specific RNA viral promoters (e.g., SP6 promoter) using the corresponding RNA polymerase (e.g., SP6 RNA polymerase) in the presence of radioactive ribonucleotides rNTP.

The probes can be labeled using radioactive nucleotides in which the isotope resides as a part of the nucleotide molecule, or in which the radioactive component is attached to the nucleotide via a terminal hydroxyl group that has been esterified to a radioactive component such as inorganic acids, e.g., ^{32}P phosphate or ^{14}C organic acids, or esterified to provide a linking group to the label. Base analogs having nucleophilic linking groups, such as primary amino groups, can also be linked to a label.

Non-radioactive probes are often labeled by indirect means. For example, a ligand molecule is covalently bound to

the probe. The ligand then binds to an anti-ligand molecule which is either inherently detectable or covalently bound to a detectable signal system, such as an enzyme, a fluorophore, or a chemiluminescent compound. Ligands and anti-ligands may be varied widely. Where a ligand has a natural anti-ligand, namely ligands such as biotin, thyroxine, and cortisol, it can be used in conjunction with its labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

Probes can also be labeled by direct conjugation with a label. For example, cloned DNA probes have been coupled directly to horseradish peroxidase or alkaline phosphatase, (Renz. M., and Kurz, K. A Colorimetric Method for DNA Hybridization. *Nuc. Acids Res.* 12:3435-3444, 1984) and synthetic oligonucleotides have been coupled directly with alkaline phosphatase (Jablonski, E., et al., Preparation of Oligodeoxynucleotide-alkaline phosphatase Conjugates and Their Use as Hybridization Probes. *Nuc. Acids. Res.* 14:6115-6128, 1986, and Li P., et al., Enzyme-linked Synthetic Oligonucleotide probes: Non-Radioactive Detection of Enterotoxigenic Escherichia Coli in Faecal Specimens. *Nuc. Acids Res.* 15:5275-5287 (1987).

Enzymes of interest as labels will primarily be hydrolases, such as phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescers include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol.

30

Sample Collection

Microbial specimens for use in this invention can be obtained from any source suspected of harbouring bacteria. The sample collection means should be uniform and reproducible such that meaningful comparisons can be made.

The samples are generally dispersed in a measured amount of buffer, though dispersal may be optimal if lysis is immediately possible. This dispersal buffer generally provides

a biologically compatible solution. A typical dispersal buffer solution would be 150mM NaCl, 20mM Tris-HCl (pH 7.5), 10mM EDTA, 10mM ethylene glycol-bis (β -aminoethyl ether) N,N,N',N"-tetraacetic acid (EGTA) or 150mM NaCl, 20mM NaPO₄ (pH 7.5), 5 10mM EDTA, 10mM EGTA. Samples may be frozen until use.

Prior to quantification, samples suspected of containing bacteria are first subjected to a lysing solution to release cellular nucleic acids. Dispersal of the sample prior to lysis is optional. Lysing buffers are known in the art. EP 10 199,439; Potts, T.V. and Berry, Em. Internat. J. Sys. Bacter., 33:765-771 (1983); Bonta, Y., et al., J. Dent. Res., 64:793-798 (1985). Generally, these buffers are between pH 7.0 and 8.0, and contain both chelating agents and surfactants. Typically, a lysing solution is a buffered detergent solution 15 having a divalent metal chelator or a buffered chaotropic salt solution containing a detergent (such as SDS), a reducing agent and a divalent metal chelator (EDTA). The use of enzymes such as N-acetyl-muramidase (lysozyme) or proteases (such as Protease K) will facilitate lysis and offer high quality 20 results.

The sample may be directly immobilized to a support or further processed to extract nucleic acids prior to immobilization. Released or extracted bacterial nucleic acid (including target nucleic acid) are fixed to a solid support, 25 such as cellulose, nylon, nitrocellulose, diazobenzyloxymethyl cellulose, and the like. The immobilized nucleic acid can then be subjected to hybridization conditions.

Alternatively, samples may be collected and dispersed in a lysing solution that also functions as a hybridization 30 solution, such as 3M guanidinium thiocyanate (GuSCN), 50mM Tris (pH 7.6), 10mM EDTA, 0.1% sodium dodecylsulfate (SDS), and 1% mercaptoethanol (Maniatis, T. et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1982).

Hybridization Conditions

Various hybridization solutions may be employed, comprising from about 20 to 60% volume, preferably 30%, of a polar organic solvent. A common hybridization solution employs 5 about 50% v/v formamide, about 0.5 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as sodium citrate, Tris-HCl, PIPES or HEPES (pH range about 6-9), about 0.05 to 0.2% detergent, such as sodium dodecylsulfate, or between 0.5-20mM EDTA, 10 0.01-0.05% ficoll (about 300-500 kilodaltons), 0.01-0.05% polyvinylpyrrolidone (about 250-500 kdal), and 0.01-0.05% serum albumin. Also included in the typical hybridization solution will be unlabeled carrier nucleic acids from about 0.1 to 5 15 mg/ml, fragmented nucleic DNA, e.g., calf thymus or salmon sperm DNA, or yeast RNA, and optionally from about 0.5 to 2% wt./vol. glycine. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents, such as polyethylene glycol, anionic polymers such as polyacrylate or polymethylacrylate, or 20 polystyrene sulfonic acid and anionic saccharidic polymers, such as dextran sulfate.

An alternative hybridization solution may be employed comprising about 2 to 4M GuSCN, preferably 3M, about 0.01 to 0.1M Tris (pH range about 6.0 to 8.5), a detergent such as 25 sodium dodecyl sulfate in concentrations of about 0.1 to 5% (w/v), and about 0.01 to 0.1M EDTA. Other additives may also be included such as carrier DNA or RNA, or protein such as bovine serum albumin or gelatin. Stringency of the hybridization solution can be adjusted by the addition of about 0 to 10% formamide, usually 5%.

30 The particular hybridization technique is not essential to the invention. Hybridization techniques are generally described in Nucleic Acid Hybridization: A Practical Approach, Ed. Hames, B.D. and Higgins, S.J., IRL Press, 1987; Gall and Pardue (1969), Proc. Natl. Acad. Sci., U.S.A., 63:378-35 383, and John, Burnstein and Jones (1969) Nature, 223:582-587. As improvements are made in hybridization techniques, they can readily be applied.

The amount of labeled probe which is present in the hybridization solution may vary widely, depending upon the nature of the label, the amount of the labeled probe which can reasonably bind to the cellular target nucleic acid, and the

5 stringency of the hybridization medium and/or wash medium.

Generally, substantial excesses of probe over the stoichiometric amount of the target nucleic acid will be employed to enhance the rate of binding of the probe to the target DNA.

10 Various degrees of stringency of hybridization can be employed. As the conditions for hybridization become more stringent, there must be a greater degree of complementarity between the probe and the target for duplex formation to occur. The degree of stringency can be controlled by temperature, 15 ionic strength, pH and the presence of a partially denaturing solvent such as formamide. For example, the stringency of hybridization is conveniently varied by changing the polarity of the reactant solution through manipulation of the concentration of formamide within the range of 0% to 50%.

20 Assay test protocols for use in this invention are those of convention in the field of nucleic acid hybridization, and include both single phase, where the target and probe polynucleic acids are both in solution, and mixed phase hybridizations, where either the target or probe 25 polynucleotides are fixed to an immobile support. The assay test protocols are varied and are not to be considered a limitation of this invention. A general review of single phase hybridization can be had from a reading of Nucleic Acid Hybridization: A Practical Approach, Ed. Hames, B.D. and Higgins, 30 S.J., IRL Press, 1985, and Hybridization of Nucleic Acids Immobilized on Solid Supports, Meinkoth, J. and Wah, G., Analytical Biochemistry, pp. 238, 267-284, 1984. Mixed phase hybridizations are preferred.

35 Nucleic acids from GuSCN-lysed bacteria can be immobilized directly on to nitrocellulose or Nytran, and hybridized with the appropriate probe. The GuSCN-lysate is diluted with buffer containing formaldehyde, slotted to

nitrocellulose and heated at 80°C to denature the nucleic acids.

5 Regardless of the assay test protocol being used, the bacterial cells are to remain in contact with a hybridization solution at a moderate temperature for an extended period of time. In single phase assays, the double-stranded duplexes may be separated from single-stranded nucleic acid by S_1 nuclease digestion followed by precipitation of duplex molecules, or by selective binding to hydroxyapatite. In mixed phase assays, 10 the support-immobilized nucleic acid is introduced into a wash solution having analogous concentrations of sodium chloride, buffers, and detergent, as provided in the hybridization solution. The time period for which the support is maintained in the wash solution may vary from several minutes to three 15 hours or more.

20 Either the hybridization or the wash medium can be stringent. Typically, for mixed phase assays, it is the wash solution that most often determines the stringency and facilitates dissociation of mismatched duplexes. After rinsing 25 the support at room temperature with a dilute buffered sodium chloride solution, the support may now be assayed for the presence of duplexes in accordance with the nature of the label.

30 Where the label is radioactive, the presence of probe can be detected in a scintillation counter. More conveniently, in mixed phase assays, the substrate can be dried and exposed to X-ray film in any number of conventional autoradiographic protocols.

35 Where the label is fluorescent, the sample is detected by first irradiating it with light of a particular wavelength. The sample absorbs this light and then emits light of a different wavelength which is picked up by a detector (Physical Biochemistry, Freifelder, D., W.H. Freeman & Co., pp. 537-542, 1982).

40 Where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies; in some cases the antibody is

labeled with a radioactive probe. (Tijssen, P., Practice and Theory of Enzyme Immunoassays, Laboratory Techniques in Biochemistry and Molecular Biology, Burdon, R.H., van Knippenberg, Ph.H., Eds., Elsevier, pp. 9-20, 1985.)

5 One method of detection is enzymatic detection in conjunction with biotin. Although fluorescence is an alternative label, enzymatic labels, in combination with avidin or streptavidin such as biotinylated peroxidase or alkaline phosphatase, are preferred. Enzyme-conjugated avidin or
10 streptavidin can also be used to directly bind the enzyme to the probe (Haase, et al., supra). Preferred enzymes are peroxidase or alkaline phosphatase. An especially preferred method utilizes enzymes directly conjugated to probes. The preferred enzymes are alkaline phosphatase and peroxidase.
15 Methods for conjugating enzymes to oligonucleotides are known. Nucleic Acid Res., 14:6115-6128 (1986) and Nucl. Acid Res., 15:5275-5287 (1987).

In the preferred instance, the UP9A assay protocol is a sandwich-type assay. A primary component of a sandwich-type assay is a solid support. The solid support has adsorbed to it or covalently coupled to it immobilized nucleic acid probe that is unlabeled and complementary to one portion of the rRNA sequence. Preferred are those probes that hybridize to regions of the ribosomal RNA with minimal secondary and tertiary interactions, such as those listed in Table 1. The advantage of such probes is that the hybridization can be carried out without the additional step of heat denaturing the sample nucleic acid. The test sample suspected of containing bacteria is then contacted with the solid support in a hybridization medium. Finally, a second soluble-labeled probe complementary to a different sequence of the rRNA of the pathogenic bacteria is hybridized to the rRNA that has formed a hybridization duplex with the immobilized nucleic acid probe on the solid support. As previously stated, the UP9A probe may function as
35 either a capture or signal probe.

Alternatively, the assay format may be a mixed phase, non-sandwich type assay. In the preferred mode, the entire assay takes place at room temperature. The bacterial sample is

lysed in the lysis/hybridization solution which contains one Nytran capture filter and biotinylated signal oligonucleotides. The hybridization is complete in 40 minutes with vigorous shaking (optional). The filter is washed free of hybridization solution and allowed to bind with streptavidin-HRP for 5 minutes with vigorous shaking. The filter is again washed, then placed in development solution for 10 minutes with gentle shaking. Color development is stopped by a final wash and the filter evaluated.

10 It is also feasible to combine the universal UP9A probe with genus, species or strain specific oligonucleotide probes to provide assays capable of quantifying the amount of specific species of bacteria rather than total bacterial count.

15 Standards

The proportion of UP9A bound to a matrix of bacterial rRNA will increase predictably and reproducibly with the amount of bacterial rRNA in the matrix. To accurately quantify the amount of rRNA present in a sample, one has to prepare 20 standards for comparison. Virtually any label or detection means of use in nucleic acid hybridizations can be standardized and quantified for use with the UP9A probe.

The standards are prepared by taking known quantities of bacteria harboring the UP9A complementary sequence and using 25 such bacteria as a control to compare the intensity of the hybridization signal to the unknown samples. The quantity of signal must correlate with the amount of hybridization such that comparison between the standard and unknowns is possible. For example, the intensity of an autoradiogram can be used to 30 compare relative amounts of hybridization. Typically, a densitometer is used for comparisons. The use of an enzyme-linked probe in a colorimetric assay format would permit the use of automated systems to measure the quantity of bacteria. This is analogous to an ELISA procedure where a 35 spectrophotometer is used to determine the quantity of antigen present in an unknown sample.

Kits

Using the UP9A probe, one can construct commercial diagnostic kits for clinical laboratories. Such kits would include instruction cards and vials containing the various 5 solutions necessary to conduct a nucleic acid hybridization assay. These solutions would include lysing solutions, hybridization solutions, combination lysing and hybridization solutions, and wash solutions. The kits would also include labelled probes. The UP9A probe could be either unlabelled or 10 labelled depending on the assay format. Standard references for comparison of results would also be necessary to provide an easy estimate of bacterial numbers in a given solution. Depending upon the label used additional components may be 15 needed for the kit, e.g., enzyme labels require substrates.

Diagnosing Periodontal Disease by Total Bacterial Count

Using standard culturing procedures the following parameters were developed which permit the diagnosis of periodontal disease using total bacterial count. Total 20 bacterial count is sometimes referred to as "bacterial load."

In a previous and unrelated study by the Forsyth Dental Center, it was reported that pockets of healthy/plaque-free or post treatment patients contain between 1×10^2 to 1×10^5 bacterial cells. Since we could not find any evidence in the 25 literature that anyone has ever suggested that a test for total bacterial load could be useful for the diagnosis of periodontal disease, it was decided to determine the total bacterial load as well as individual pathogenic periodontal bacteria in normal, diseased-, diseased-after treatment with tetracycline 30 fibers and diseased-patients after scraping of the teeth by cultural procedures. Appropriate plaque samples were collected by curette and deposited in culture media and the cell numbers for total bacteria and for the individual pathogenic bacteria 35 were determined by established microbiological techniques as shown in Table 2.

Table 2. Average number of total and individual bacterial numbers determined by culture for diseased pockets at the Forsyth Dental Center and the University of Washington.

		FORSYTH STUDY ^a	U.W. STUDY ^b
5	Total bacteria	6×10^7	7×10^7
<hr/>			
	Individual bacteria		
	Actinobacillus		
10	<i>actinomycetemcomitans</i> (Aa)	2×10^6	5×10^6
	<i>Bacteroides gingivalis</i> (Bg)	4×10^6	7×10^6
	<i>Bacteroides intermedius</i>	6×10^6	8×10^6
	(Bi)	3×10^6	4×10^6
	<i>Eikenella corrodens</i> (Ec)	5×10^5	2×10^5
15	<i>Fusobacterium nucleatum</i>	1×10^6	3×10^4
	(Fn)		
	<i>Wolinella recta</i> (Wr)		
<hr/>			
	a) 100 pockets		
20	b) 76 pockets		

Table 3. Total bacterial numbers determined by culture for diseased, treated and normal pockets at the Forsyth Center and the University of Washington.

	Disease State	Total bacterial numbers
	Disease(100) ^a	6×10^7
	Severe/moderate ^b	7×10^7
	Post treatment(40) ^a	2.5×10^6
30	Normal(20) ^b	5×10^6

a) Determined at the Forsyth Center.
 b) Determined at the U.W.

35 The following conclusions can be made from the data in Tables 2 and 3:

a) That there is about a 90% drop in the average cell numbers going from the diseased to the normal state.

b) A similar drop in cell numbers is observed when the diseased states are treated with either tetracycline or after scaling.

5 c) It appears from these two studies that when plaque samples are collected with a curette the diseased state starts when total bacterial cell numbers increase substantially over 5×10^6 cells.

10 As more data is accumulated these values will be refined. It should be pointed out that about 10 fold more plaque sample is collected with a curette scrape then with a paper point. Therefore the cutoff cell number for the determination of disease state will depend on the sample collection procedure.

15 Accumulated evidence exists which confirms a correlation between total bacterial numbers and the state of bacterial vaginosis. Therefore it is possible to determine the total bacterial numbers in the same way as for periodontal disease.

20 It is known that the progression of bacteremia depends on the total bacterial cell counts. Similarly the total bacterial cell numbers could be determined in the same way as for periodontal disease.

25 It is observed with peptic ulcers that the pH of the stomach increases and favors the increased growth of bacteria. We therefore believe that the total bacteria number may be indicative of the presence of ulcers. The total bacterial test may therefore also be used in this case.

30 Example 1: Detection of Total Bacteria in Heated GuSCN Lysate Using a Colorimetric Sandwich Assay Format and UP9A-Nytran as a Capture System

35 A lysis solution composed of 3 M GuSCN, 2% Sarkosyl, 50 mM Tris, pH 7.6, 25 mM EDTA was used to lyse a mixture of 1×10^8 cells of Aa, Bg, Bi, Ec, Fn and Wr in 100 microliter volumes at 19°C. The lysate was then heated in a 65° water bath for 10 minutes. Biotinylated 24-mer oligonucleotide probes (UP7B and UP3A) complementary to conserved regions of

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